



NO synthase? Generation of nitric oxide in plants

JOHN T. HANCOCK

Faculty of Health and Life Sciences
University of the West of England
Coldharbour Lane
Bristol, BS16 1QY, UK

Correspondence:

Dr John T. Hancock
Faculty of Health and Life Sciences
University of the West of England
Coldharbour Lane
Bristol, BS16 1QY, UK
Email: john.hancock@uwe.ac.uk

Abbreviations:

ABA	– abscisic acid
BH ₄	– tetrahydrobiopterin
CuAO1	– copper amine oxidase1
DAF	– diaminofluorescein
EDRF	– endothelial derived relaxing factor
eNOS	– endothelial nitric oxide synthase
FH ₄	– tetrahydrofolate
iNOS	– inducible nitric oxide synthase
L-NAME	– N(G)-nitro-L-arginine methyl ester
nNOS	– neuronal nitric oxide synthase
NO	– Nitric oxide
NO	– nitric oxide synthase
PAs	– polyamines

Key words: nitrate reductase; nitric oxide; nitric oxide synthase, reactive oxygen species; signal transduction

Abstract

It has now become well accepted that nitric oxide (NO) has a key role to play in the signalling that takes place in plant cells. However, the sources of NO in plants has been hard to determine and there is considerable debate as to exactly how NO is made by plant cells. In animals nitric oxide synthase (NOS) enzymes have been characterised and such data has been used to inform the studies which have been taking place in plants. However, despite several genomes from higher plants being sequenced, there is no evidence that such species contain NOS sequences. Despite this, a recent search using algal sequences did reveal a NOS-like sequence and such a finding may spark new enthusiasm for the search for a higher plant NOS. However, considerable care needs to be taken in such studies, as the robustness of many of the inhibitors and probes which could be used in such work has been questioned. Here, some of the previous evidence that has been presented for the existence of a plant NOS, along with a discussion of how else plants may produce NO is given.

THE GENERATION OF NO IN PLANTS

Recent publication of a paper reporting the presence of a nitric oxide synthase (NOS) in algae by Foresi *et al.* (1) has perhaps opened the way for more speculation of whether plants do indeed contain an enzyme analogous to that found in mammals. The mammalian system was characterised during the 1990's, while it was reported that plants use nitric oxide as a signal in 1998 (2, 3). However, there is still controversy as to whether higher plants really contain a true NOS enzyme. Here I will discuss some of the background to this controversy and which led to the paper showing that some algae at least contain a NOS protein (1).

Nitric oxide (NO) was first reported to be important in plants back in 1998 by two groups (2, 3). Since then there has been a flurry of activity, with a view that the information gleaned from work in the animal kingdom should be able to inform such studies on plants, perhaps accelerating the latter to a point where it can catch up. However, this was not to be so easy as first envisaged.

NO as a signal in animals was first mooted by Moncada's group in 1987(4). It was suggested that endothelial derived relaxing factor (EDRF), which was produced by endothelial cells, and caused relaxation of smooth muscle cells, was in fact the relatively reactive gas nitric oxide. On first appearance this seems a little odd. NO is a gas, but it does dissolve in aqueous solution. However, NO is usually found as a free radical, so it is quite reactive. In fact the chemistry of NO is a little more complex with forms such as NO⁺ existing as well as the classical radical

form NO. But, if this unusually chemical was to be a true biological signal it had to be fit into a series of generic principles. Namely, it has to be made where and when needed, it needs to be able to move to its place of action, it needs to be recognised as a specific signal when it gets there, and it needs to be efficiently removed once it has completed its role. Taking the last point first, removal of NO is at first sight relatively easy because its inherent reactivity will mean that it will rapidly become oxidised to nitrite and nitrate. Movement of NO has always been assumed as it is small, relatively soluble in water, and also soluble in lipids and hydrophobic environments, and it is not charged in its radical form so will be able to cross membranes, even if they have an electrical potential across them. However, this simplistic view has been challenged by some, with membranes being thought to accumulate NO and affect its reactivity [5]. But one of the main challenges is to determine how the gas is made by biological samples in the first place.

The isolation of a synthase from animal tissues which could make NO was surprisingly rapid with early reports isolating an enzyme that was calcium and calmodulin dependent (6). Since then it has been discovered that humans have three genes which encode NOS. There is the endothelial enzyme (eNOS), a neuronal version (nNOS) and an inducible enzyme (iNOS) which was originally isolated from macrophages and was named mNOS. Such enzymes are now eagerly studied as drug targets (7).

With this in mind, it seemed easy to find such an enzyme in plants. The enzyme was being well characterised in animals, and it was found to have similarities to the enzyme P450 reductase. It was known to use L-arginine as a substrate, to have a hydroxyl-arginine intermediate, and to produce citrulline and NO. The reaction needed the input of electrons from NADPH and the presence of tetrahydrobiopterin (BH₄). In plants reports did start to appear that suggested that there was such an enzyme to be isolated. However, such early papers were debated (8) and further work found that NOS in plants was not as easy to find as it was thought. One of the early enzymes to be suggested to have NOS activity was later found to have GTPase activity (9), and it was suggested that it was renamed to be called AtNOA1. Such an enzyme may be required for NO generation, but it was not the synthase *per se*.

Many groups became sceptical about the existence of a NOS in plants and reports have appeared of alternative enzymes which could be involved in NO generation in plants (see below). However, the plant NOS story is far from over, and with the discovery of an early plant having a NOS-like gene (1) then the search may start in earnest again. The question to be asked is should this search be undertaken, or are groups chasing shadows?

The NOS with which I started this discussion was found in algae (1). Using the NOS sequence as a starting point the authors reported that they could find two such sequences in the *Ostreococcus* genus, specifically in *O. tauri* and *O. lucimarinus*. Concentrating on that of *O.*

tauri it was found that the amino acid sequence of the NOS is 45% similar to that of a human NOS, and that its folding was likely to be similar to that of human iNOS. A K_m for L-arginine was found to be 12 ± 5 µM for the purified recombinant *O. tauri* NOS and over-expression of recombinant *O. tauri* NOS in *Escherichia coli* cells showed increased levels of NO. This is a significant finding but it does have one caveat. This organism belongs to a primitive class within the green plant lineage, the Prasinophyceae (Chlorophyta), and there is no reason to suspect that higher plants have retained this gene. In fact since the full genomes of plants such as *Arabidopsis* have been known for some time (10) it is a surprise that NOS has not been found if it does in fact exist. The reaction which it undertakes is quite complex, and requires the involvement of binding sites for cofactors and redox prosthetic groups, such as flavins and haem groups, so it is possible that the enzyme is a multi-peptide complex, unlike the mammalian version, but even this is seemingly more unlikely as time fails to reveal likely candidates.

However, there are regular reports that NOS does exist in plants. Some of the evidence is based on assays of NOS-like activity. These follow the production of citrulline from arginine, with the assumption that NO is generated at the same time. However, it was reported that in plants the assay for the citrulline itself is unreliable, as there was interference from argininosuccinate, generated by argininosuccinate lyase (EC 4.3.2.1). The authors suggested that the generation of citrulline needs to be verified if such assays are to be useful (11) but such care is rarely exercised.

There is other evidence too. Recently NOS was implicated in auxin mediated root branching (12). In this study NOS was suggested as the root branching was reduced by a NOS inhibitor, L-NAME. Such inhibitors were originally used in animal work as they are analogues of L-arginine, the NOS substrate. However, there is little concrete evidence of exactly what such inhibitors are doing in plants. Without a specific protein target it is making an assumption that the NOS peptide is there to be inhibited. Many such reports are in the literature (*eg* 13, 14) and it will be interesting in the future to find out the exact action of such inhibitors.

Peroxisomes are thought to contain NOS activity (15), again being inhibited by NOS inhibitors. Under stress conditions NOS activity in the peroxisomes influenced the NO accumulation in the cytoplasm, while the NOS proteins appeared to require the action of the peroxins Pex12 and Pex13 for transport of the NOS protein into the peroxisomes. In a further paper by the same group they gave further evidence as to the existence of NOS in peroxisomes (16). They suggested that as there is evidence of the presence of a NOS from at least eleven different plant species then the support for a real plant NOS is great enough to believe in its existence. They further suggest that as NO generation is affected in some mutants with an imbalance in L-arginine metabolism then this can be taken as further evidence for the exi-

stence of a NOS enzyme. On discussing the evidence that plants lack the vital cofactor BH₄ they cite the report that BH₄ may be substituted by tetrahydrofolate (FH₄) whose biosynthesis distribution is well known in higher plants (17), and they conclude that an L-arginine-dependent NOS activity is in at least two subcellular compartments, that is the peroxisomes (16) and chloroplasts (18).

Evidence for NOS in peroxisomes has also recently been reviewed by Del Río (19). Here evidence of NOS in different species is tabulated and includes work reported in *Lupinus albus*, *Nicotiana tabacum*, *Pisum sativum* and eight other species. Further evidence cites a specific activity of a pea leave peroxisome NOS as being 5.6 nmol mg⁻¹ protein min⁻¹ when measured as citrulline production, and a list of inhibitors that reduce such activity. The enzyme was said to be dependent on L-arginine and NADPH while it required calcium ions, calmodulin, BH₄, FAD and FMN, just like the mammalian NOS enzyme family. But none of this evidence is backed up by any definitive identification of a protein or gene to encode such a protein.

If there is no widespread existence of NOS in plant cells then the NO that has been reported has to be able to be made by other means. One of the main enzymes thought to be involved is nitrate reductase (NR). NR is usual thought of as being part of the nitrogen assimilation pathways in plants, an immensely important aspect of plant physiology (20). NR was also found to be able to generate NO both *in vitro* and *in vivo* (21–23). This was not just an odd phenomenon but thought to have physiological relevance. NR was shown to be involved in the NO generation seen during the control of stomatal closure (24), to be important during flowering (25) and to be involved in nitric oxide production in *Medicago truncatula* nitrogen-fixing nodules (26) for example. One of the ways in which NR can be implicated in NO generation is by the addition of tungstate, which reduces NR activity. This was carried out by Chen and Kao (27) for example when they reported that NR was important for NO production in response to indole-3-butyric acid. Plants such as *Arabidopsis* have two genes for NR and therefore the question which is often the focus of attention is which of these is most important for NO generation. NR was found to be important in cold responses (28). In *Arabidopsis thaliana* following 1–4 hours of chilling NO production was detected but this was impaired in a *nia1nia2* nitrate reductase mutant, in which the both NR enzymes would be affected. NR dependent NO was also found to be important in cold and freeze tolerance by Zhao *et al.* (29), again taking advantage of the *nia1nia2* mutants. In a similar manner it was found that NR was involved in the osmotic stressed induced NO generation in roots (30). Again *nia1nia2* mutants were used, but the role of each isoform was not defined. It was also found, once again using the double *nia1nia2* mutant, that NR was important for NO synthesis during plant defenses against pathogen attack (31).

However the involvement of the individual NR enzymes was still not unravelled in these cases above. Others though have looked at the enzymes separately. Hao *et al.* (32) investigated the downstream effects of salicylic acid (SA), with particular reference to NO. Effects were significantly reduced if NO scavengers were used, but also reduced if inhibitors of either NOS or NR were added. They then used NR mutants, either single mutants of *nia1* or *nia2*, or the double mutant *nia1nia2* and showed that in fact in this system both NR enzymes are involved. However, specific roles for the enzymes can be determined. ABA-induced stomatal closure appears to be dependent on the synthesis of NO by the *NIA1*-encoded isoform, NR1 (33, 34). Recently using a fungal elicitor PB90 (a protein elicitor from *Phytophthora boehmeriae*) it was found that such a treatment of cells from *Camptotheca acuminata* stimulated NR activity and induces up-regulation of *NIA1* but there was no effect on *NIA2* expression in the cells (35).

Therefore clearly NR is an immensely important enzyme to make NO during a range of physiological responses in plants, with perhaps NR1 being more important than NR2 for a NO signalling role in some cases. But other enzymes have been suggested to be involved too. Working on roots Stöhr *et al.* (36) suggested the presence of a PM-bound nitrite: NO-reductase (NI-NOR) which was found to be insensitive to cyanide and unique to NR. It was around 310kDa and was thought to reduce apoplastic nitrite. On the other hand Tun *et al.* (37) found that the polyamines (PAs) spermidine and spermine increased NO release in the *Arabidopsis* seedlings. Recently it has been reported that in *Arabidopsis thaliana* copper amine oxidase1 (CuAO1) mediates the generation of NO in response to both abscisic acid (ABA) and polyamine. One further enzyme to be implicated is xanthine oxidoreductase, which can produce NO under near anaerobic conditions (39), but there is little evidence of a major involvement in plants.

Lastly it should not be forgotten that NO can be produced in plants in an enzyme-independent manner (40). So in some cases the presence or absence of an enzyme such as NOS may be an irrelevance.

DETECTION OF NO AND ESTABLISHING SOURCES

In any future research which aims to find sources of NO in plants there will be a need to robustly measure the presence of NO, and in particular the production of NO by a isolated purified enzyme. Both in animal and plant research one of the ways used to measure the presence of NO is the Griess assay (41). However, the assay does not measure NO itself, but a downstream product, and there needs to be care to be sure that the measurements actually reflect the NO that was present originally. Other methods are based on haemoglobin, which relies on the conversion of oxy-haemoglobin to met-haemoglobin. This was used by Moreau *et al.* for example (9). Other methods include the use of ozone based chemiluminescence.

ce (42, 43), although the majority of reports seem to use fluorescence based methods. These are commonly reliant on diaminofluorescein (DAF)-based probes [for example 39], but there are also rhodamine-based probes available too (44). Some of these dyes are supplied as a diacetate ester so will be accumulated in the cells and hence can give an indication of the subcellular location of NO, which is extremely useful. However caution is also needed here because the fluorescent product formed when DAF reacts with NO can also be moved post generation. Usually the location is visualised with a confocal microscope, but by the time the sample is studied it is possible that the fluorescent materials created have moved, perhaps being accumulated in particular parts of the cell. Alternatively, extracellular NO can be determined by the non-acetate versions of the dyes, and this may be useful if plant extracts suspected of containing NO synthesising proteins are being investigated. But despite the common nature of the use of such dyes there are great concerns about what they are exactly measuring. For example (45, 46) it has been suggested that changes in DAF fluorescence do not necessarily reflect NO production, but may be caused by NO oxidation or the generation of other DAF-reactive compounds.

Other methods include laser photoacoustic techniques (47) which can be used for *in planta* samples, but the gold standard is the use of electron spin resonance (ESR; otherwise known as electron paramagnetic resonance

(EPR)) [see 42 for example]. However, this relies on the formation of NO-derived products, is extremely expensive, and would not give any indication of the subcellular location of the NO generation. For that latter reason, DAF-based dyes are still often the method of choice for *in planta* work.

However, all of these methods yield data that needs to be treated with some caution and it is best to confirm data with two independent methods – although this seems to be rarely done. It is also preferable too to repeat experiments in the presence of a NO scavenger with the most common used being 2-phenyl-4,4,5,5-tetramethylimidazole-1-oxyl 3-oxide (PTIO) or its derivatives (48).

DOWNSTREAM OF NO GENERATION

Once NO is produced, despite the mechanism, there has to be a way for it to be perceived by components of the cell and to have an effect. Only a brief description of possible events will be given here, but it should be noted that downstream effects are not always easy to determine. Unlike plant hormones such as ethylene there seems to be no specific receptors for NO, and the existence of such proteins has not actively been sought. However, NO downstream targets of NO are being identified in plants. NO effects are often through effects of protein associated iron or by covalent modification of amino acids within the proteins.

In mammalian cells in response to NO cGMP is increased, produced by soluble guanylyl cyclase, a haem containing protein, which makes it a target for NO. In plants cGMP has been implicated in downstream signalling too (49, 50). Although it is not easy to measure small transient changes in cGMP in plant cells new methods are being developed (51). Other NO effects include S-nitrosylation of plant proteins and such modifications of proteins in plant cells can be determined (52). In a similar way to nitrosylation there are other covalent modifications to proteins which can be mediated by NO including nitration (53) and such mechanisms should not be ignored. NO may also react with reactive oxygen species with the generation of peroxynitrite and so NO may be instrumental in initiating peroxynitrite signalling too (54).

However the exact downstream events which are brought into play by the presence of NO will be determined by where and to what level NO is produced, and this will be determined by the NO producing mechanisms which are in place. Furthermore, they will not necessarily be the same in all places of the cell, so robust determination of NO sources needs to be undertaken before a full understanding of NO signal transduction pathways is obtained.

CONCLUSION

Nitric oxide is undoubtedly important as a signal in plants (see Figure 1), and has been implicated in many physiological events, including root branching (12), stomatal closure (24), flowering (25), root nodule function

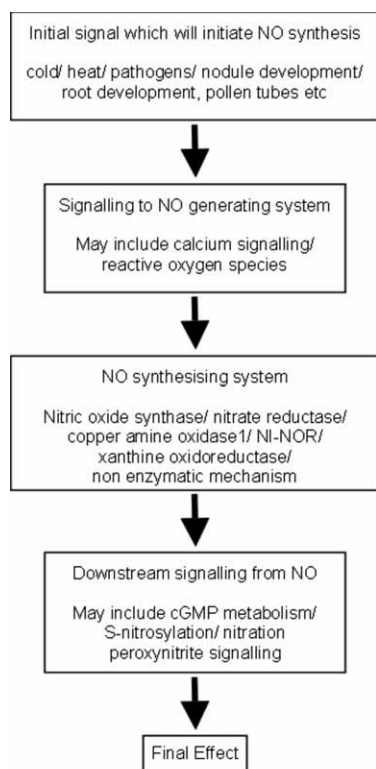


Figure 1. Nitric oxide production in plants. Such generation needs to be initiated, have an enzyme or source of NO, and NO needs to have a downstream effect.

(26) and root gravitropic bending (55) to name a few. NO signalling has recently been reviewed by others (56). There are many potential NO sources in plants, again reviewed by others (57). However, with the publication of an algal NOS sequence (1) it is probable that new investigations into the presence of NOS in higher plants will be initiated. Care needs to be exercised in such studies. The search for NOS in plants has already sparked debate (8) with false starts and raised hopes. Perhaps the best evidence of a NOS is in peroxisomes and such work appears to be continuing (16, 19). However there are other likely sources of NO such as NR (22) which are going to be important to understand. There is evidence of NO generation in organelles such as chloroplasts (18) and mitochondria (58) although the NO metabolism which takes place in the latter is not easy to comprehend (58).

At the end of the day it is most likely that plant cells will contain many ways to generate NO, but it will be the levels to which it is produced, and the location at which it is produced which will be most critical to the downstream signalling that ensues. Such signalling pathways will also be under the influence of other compounds being generated at the same time, such as reactive oxygen species, so as well as the determination of whether plants truly have a NOS enzyme or not, NO signalling needs to be put into context of all the other signalling which is taking place at the same time.

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